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(54) Title: VIRAL VECTOR SYSTEM CAPABLE OF EXPRESSING AN APOPTOSIS–ASSOCIATED GENE

(57) Abstract

An apoptosis–resistant virus–sensitive cell is disclosed. This cell has an apoptosis resistance gene, such as crmA, bcl–2, bcl–x1, FLIP, survivin, IAP, or ILP gene. The generation of adenovirus vectors capable of expressing apoptosis–associated genes is achieved using said cell.

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DESCRIPTION

VIRAL VECTOR SYSTEM CAPABLE OF EXPRESSING AN APOPTOSIS-ASSOCIATED GENE

Technical Field

This invention relates to viral vector systems capable of expressing apoptosis-associated genes. More particularly, it relates to recombinant viruses containing apoptosis-associated genes, and systems for generating such viruses.

Background Art

In addition to congenital diseases, a great variety of acquired diseases have become the object of gene therapy. In order to transfer objective genes, vectors designed variously according to the target cell and the objective gene have been proposed. It may safely be said that these designs are being made principally from the viewpoint of gene transfer efficiency and safety.

However, for some objective genes to be transferred, recombinant viruses cannot always be generated efficiently or cannot possibly be generated. More specifically, in generating a recombinant virus by the infection of a certain virus-sensitive cell with a virus or the transfection thereof with an expression plasmid, it will be very difficult to obtained the desired recombinant virus if the expression of the objective gene integrated into the virus causes cell damage (in particular, apoptosis) to the aforesaid cell.

Actually, in order to generate a recombinant adenovirus containing an apoptosis-associated gene as the objective gene, the present inventors carried out a conventional method using the ordinary 293 cell line, but failed to obtain a recombinant adenovirus capable of

expressing the FAS (CD 95), Fas Lig, Bcl-2, Bcl-2 antisense or Bax gene. The reason for this seems to be that, if an adenovirus vector capable of expressing an apoptosis-associated gene is introduced into animal cells, the cells producing the virus will be destroyed because the period of time required to induce cell death by apoptosis is shorter than that required to replicate and produce the virus, resulting in failure to obtain a recombinant virus having an apoptosis-associated gene integrated thereinto.

There are a variety of diseases for which the induction of apoptosis by gene transfer or, on the contrary, the inhibition of harmful apoptosis by gene transfer serves as an effective therapeutic means. Accordingly, a need for a means which enables recombinant viruses capable of expressing apoptosis-associated genes to be generated efficiently will continue to exist.

Disclosure of Invention

The present inventors have now found that the above-described problem (i.e., the inability of the conventional method to generate a recombinant virus capable of an apoptosis-associated gene) can be solved by establishing an apoptosis-resistant 293 cell line. In addition to the 293 cell line, the establishment of such cell lines can likewise be applied to other cell lines usable as producer cells, owing to their mechanism of action.

Thus, the present invention provides an apoptosis-resistant virus-sensitive cell line having an apoptosis resistance gene introduced thereinto. By using this cell line as a producer cell, a virus having an apoptosis-associated gene integrated thereinto in an expressible form can be generated efficiently.

Accordingly, the present invention also provides

a method for the generation of a recombinant virus containing an apoptosis-associated gene, and such recombinant viruses. By properly choosing the genes contained therein, such recombinant viruses are useful as vectors for gene therapy which can be applied to cancer therapy for destroying cancer cells selectively, the treatment of autoimmune diseases and graft rejection reactions, and apoptosis induction therapy for inflammatory cells in inflammatory diseases.

Brief Description of Drawings

Fig. 1 is a photograph substituting for a figure to show that 293/crmA cells are resistant to transfection with pAxCAhFasLig plamid DNA. This photograph exhibits microscopic observation (magnification: 100) of the state of cells on the day after the transfection. 293 cells of parent strain suffered serious damage from the transfection (See: photograph A). In contrast, 293/crmA cells received almost no damage (See: photograph B). Transfection was conducted in the following manner: 293 cells and 293/crmA cells were inoculated each 40,000 in number in a 6 cm dish, and, two days later, were each transfected with 0.016 mg of pAxCAhFasLig plamid DNA by means of calcium phosphate method.

Fig. 2 is a graph to show that strong expression of FAS is caused by the infection with recombinant adenovirus AxCAhFAS. As a control, U251 cells were infected with AxCAlacZ at an moi of 100. Anti-human CD 95-FITC (Pharmingen) was used as a primary antibody in an amount of 10 μℓ per 1 x 10^6cells, and anti-Leu 2a (anti-CD 8)-FITC (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used as a control antibody in an amount of 10 μℓ per 1 x 10^6cells. It was confirmed that the expression of FAS in U251 cells was very greatly enhanced by infection with the recombinant adenovirus

AxCAhFAS (Peak 4). Peak 1 shows U251 cells stained with the control antibody. Peak 2 shows uninfected U251 cells stained with anti-human CD 95-FITC (Pharmingen). In U251 cells, a slight expression of FAS is observed. Peak 3 shows U251 cells infected with AxCAlacZ as a control. Although infection with the simple adenovirus causes a slight change in the expression level of FAS, said change is very little as compared with the change shown by peak 4.

Fig. 3 is a graph to show that strong expression of FAS ligand is caused by the infection with recombinant adenovirus AxCAhFasLig. It was confirmed that the expression of FAS ligand in U373 cells was induced by infection with the FAS ligand adenovirus (AxCAhFasLig). Peak 1 shows the results of analysis of control U373 cells, and peak 2 shows the results of analysis of U373 cells infected with the FAS ligand adenovirus (AxCAhFasLig).

Fig. 4 is a micrograph (magnification: 100) substituting for a figure to show the state of subline of U251 (U251-FAS-H) two days after infected with AxCAhFADD. When growing, U251 cells inherently keep adhered to a plastic plate. In this micrograph, however, most of the cells are caused to float on the surface by the infection with AxCAhFADD.

Fig. 5 is a photograph substituting for a figure to exhibit the results of western blot analysis (gel filtration electrophoresis) which show that human FADD is strongly expressed on account of the infection with adenovirus AxCAhFADD. Human glioma cells U373 were infected with the adenovirus AxCAhFADD at moi of 100, and, two days after the infection, proteins were extracted from the cells and were then subjected to western blot analysis. Lane 1 shows uninfected control U373 cells. Lane 2 shows control U373 cells infected with AxCA-lacZ at moi of 100. Lane 3 shows the results of

western blot analysis of proteins extracted from U373 cells which had been infected with AxCAhFADD at moi of 100. These analyses were carried out according to an ordinary procedure with use of a commercially available anti-FADD antibody (Santa-Cruz, polyclonal anti-FADD antibody).

Specific Description of the Invention

In the context of the present invention, the term "apoptosis resistance gene" denotes any gene that, when a producer cell is generated by introducing the gene thereinto, can impart thereto resistance to apoptosis due to the expression of an apoptosis-associated gene, without regard to the type thereof. As used herein, the expression "can impart thereto resistance to apoptosis" means the ability to modify the producer cell in such a way that, when a virus containing an apoptosis-associated gene is introduced thereinto, the producer cell can survive for a longer period of time than that required to replicate and produce the gene. Examples of such apoptosis resistance genes include, but are not limited to, the crmA gene [cowpox virus white-pock variant (CPV-W2) (crmA): Pickup, D.J. et al., Proc. Natl. Acad. Sci. U.S.A. 83, 7698- 7702 (1986); J. Virol. 63, 4632-4644 (1989)], the bcl-2 gene [Reed, J.C., J. Cell Biol. 124, 1-6, 1994], the bcl-xl gene [Boise, L.H. et al., Cell 74(4), 597-608 (1993)], the FLIP gene [human FLICE-like inhibitory protein: Irmler et al., Nature 388, 190-195, 1997], the survivin gene [IAP-like apoptosis inhibitor: Ambrosini et al., Nature Medicine 8, 917-921, 1997], the IAP gene [baculovirus apoptosis-inhibiting protein: Boise et al., J. Virol. 67, 2168-2174, 1993] and the ILP gene [IAP-like protein ILP: Duckett et al., EMBO J. 15(11), 2685- 2694, 1996].

The cell from which the producer cell is derived may be any virus-sensitive cell that meets the purpose

of the present invention. However, an especially preferred example thereof is the 293 cell line used for the multiplication of adenoviruses. For example, in the case of infection with an adenovirus, it is preferably to use a properly modified cell line, such as one having introduced thereinto the E1 gene which is usually the first to be expressed after infection with an adenovirus [Becker, T.C. et al., Methods in Cell Biol. 43, 161-189 (1994)].

Moreover, this cell line must be infectible with viruses belonging to a family selected from the group consisting of the families *Adenoviridae*, *Retroviridae*, *Parvoviridae*, *Herpesviridae*, *Poxviridae*, *Papovaviridae* and *Hepadnaviridae*. Preferably, this cell line must have the property of being at least infectible with human adenoviruses belonging to the family *Adenoviridae*.

Specific examples of the above-defined cell line include 293/crmA, 293/bcl-2 and 293/bcl-xl.

According to the present invention, a recombinant virus containing (or having integrated thereinto) an apoptosis-associated gene can be efficiently generated (or produced) by using a cell line (or producer) as described above. This may be accomplished by providing an apoptosis-resistant cell line as described above; infecting or transfecting cells of this cell line with a virus (or expression vector) having an apoptosis-associated gene integrated thereinto in the presence of a gene unit capable of replicating or producing the virus, a helper virus (serving, for example, to make up for the function of the gene removed to integrate the apoptosis-associated gene) or the like, if necessary; and culturing the infected (or transfected) cells to multiply the recombinant virus. The respective steps of this procedure may be carried out according to the techniques well known to those skilled in the art (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. ed., 1989,

Cold Spring Harbor Laboratory Press, New York; Saito and Sugano (eds.), "An Extra Issue of Experimental Medicine: Experimental Methods for Gene Transfer and Expression Analysis", 1997, Yodosha]. In the case of viruses belonging to the family *Parvoviridae* (e.g., adeno-associated viruses), a helper virus such as an adenovirus is used in combination with them.

Thus, according to the present invention, a recombinant virus having an apoptosis-associated gene integrated thereinto can be obtained in large amounts. The term "apoptosis-associated gene" as used herein comprehends all genes that permit recombinant viruses to be generated in the above-described manner and used as a means of gene therapy for the treatment of diseases in animals including man. Specific examples of such genes include, but are not limited to, the FAS gene [in particular, human FAS; Ito, N. et al., Cell 66, 233- 243 (1991)], the Fas Lig gene [human Fas ligand gene; see Mita et al., Biochem. Biophys. Res. Comm. 204, 468-474], the FLICE gene [human FADD-homologous ICE/CED three-way protease gene; Muzio, M. et al., Cell 85(6), 817-827 (1996)] and the bcl-xs gene [Boise, L.H. et a., Cell 74(4), 597-608 (1993)]. These genes are inserted into a position of viral DNA from which a gene that is expressed in animal cells upon infection with the virus and functions to produce the virus has been removed in advance, according to the per se known techniques [Sato et al., Methods for the Generation of Recombinant Adenoviruses, pp. 27-42; Saito and Sugano (eds.), "An Extra Issue of Experimental Medicine: Experimental Methods for Gene Transfer and Expression Analysis", 1997, Yodosha]. Specific examples of the virus have been given above. The recombinant virus (or expression vector) so generated may be introduced into cells of the above-described cell line and multiplied therein. Thus, a recombinant virus (or vector) in accordance with the

present invention can be obtained.

As described above, preferred examples of the virus used in the present invention are human adenoviruses which are already being used as vectors for gene therapy. They also include all variants that have been modified in such a way as to meet the purpose of the present invention.

As will be described later in greater detail, recombinant adenoviruses can be generated by using the 293/crmA cell line according to one specific embodiment of the present invention and transfecting it with an adenovirus-derived plasmid pAx CA- [Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C., and Saito, I. (1996), Proc. Natl. Acad. Sci. USA 93, 1320-1324; and Niwa, H., Yamamura, K., and Miyazaki, J. (1991), Gene (Amst.) 108, 193-200] into which an apoptosis-associated gene as described above has been integrated. Specific examples of such recombinant adenoviruses include AxCA-FAS (CD 95), AxCA-Fas Lig, AxCA-FLICE (Caspase-8) and AxCA-bcl-xs.

These recombinant adenoviruses serve to introduce the objective gene into target cells in vitro, ex vivo or in vivo.

The present invention is more specifically described hereinbelow by using the 293/crmA cell line as the apoptosis-resistant virus-sensitive cell line and Ax CA-FAS (CD 95), Ax CA-Fas Lig or Ax bcl-xs as the virus (or expression vector) containing an apoptosis-associated gene. However, the following examples are not to be construed to limit the scope of the present invention.

Example 1: Generation of an apoptosis-resistant virus-sensitive cell line 293/crmA

Preparation of pRx-crmA-bsr (Kozak) from pCDNA 3 crmA:

a) pCDNA 3 crmA (obtained from Dr. David J. Pickup) was digested with *Eco*RI to excise an about 1468

bp fragment containing crmA. This fragment was cloned into the *Eco*RI site of pRx-bsr [for pRx, see Wakimoto et al., Jap. J. Cancer Res. 88, 296-305 (1997); and for the DNA sequence of bsr, see SEQ ID NO:1 that will be given later] to generate pRx-crmA-bsr (not Kozak) having crmA inserted thereinto in the right direction.

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b) On the other hand, an *Xho*I-*Nco*I fragment (about 2034 bp) was obtained from pRx-nZ (see the aforementioned article by Wakimoto et al.), an *Nco*I-*Not*I fragment (about 1220 bp) from pRx-crmA-bsr, and an *Not*I-*Xho*I fragment (about 4537 bp) from pRx-crmA-bsr. These fragments were linked together to generate pRx-crmA-bsr (Kozak).

In pRx-crmA-bsr (Kozak), the base sequence in the neighborhood of the initiation codon of crmA was designed so as to consist of the consensus sequence of Kozak. The full-length gene sequence of this pRx-crmA-bsr (Kozak) is shown as SEQ ID NO:1. In this sequence, a base sequence consisting of a large number of "A"s arranged on the outside of the LTR of the retrovirus represents an unidentified sequence part for convenience' sake.

Introduction of the crmA gene into 293 cells:

A basic method for gene transfer by use of a retrovirus vector is described in Shinoura and Hamada, "Retroviruses", pp. 58-62; and Saito and Sugano (eds.), "An Extra Issue of Experimental Medicine: Experimental Methods for Gene Transfer and Expression Analysis", 1997, Yodosha. In this example, the method described in the above references was basically employed to establish a cell line capable of expressing crmA on a high level. However, the method employed in this example was based on selection with blasticidin.

Twenty hours before transfection, 5.5×10^6 BOSC 23 packaging cells (see Pear, W.S. et al., Proc. Natl.

Acad. Sci. USA 90, 8392-8396, 1993) were inoculated in a 10 cm dish. In this case, 30 ml of a cell suspension having a density of 5.5×10^5 cells per ml was prepared and 8-, 10- and 12-ml portions thereof were inoculated on three plates. On the next day, a plate showing 80% confluency was chosen. In order to achieve a high titer, it is most important that BOSC cells are inoculated so as to be uniformly scattered without forming lumps. To this end, BOSC cells may be inoculated once more on the previous day.

800 µl of OPTI-MEM (Gibco/BRL) was gently added to 25 µl of pRx-crmA-bsr, followed by agitation (fluid A). Separately, 750 µl of OPTI-MEM was placed in a sterilized tube, and 50 µl of LIPOFECTAMINE (Gibco/BRL) (see Neumann, J.R. et al., Biotechniques 5, 444, 1987) (2 mg/ml) was slowly added thereto and suspended therein (fluid B).

Fluid A was mixed with fluid B, and this mixture (fluid C) was allowed to stand at room temperature for 40 minutes. BOSC 23 cells were washed once with warm Dulbecco modified Eagle's medium (DMEM) free of an antibiotic and fetal bovine serum (FBS), followed by the addition of fluid C to the BOSC 23 cells. Moreover, 2.4 ml of OPTI-MEM was added and the resulting suspension was incubated for 5 hours in the presence of 5% CO_2. Then, DMEM containing 4 ml of 20% FBS was added and the incubation was continued overnight. On the next day, the culture medium was replaced by warm Dulbecco modified Eagle's medium (complete) containing 10% FBS. At the same time, 2×10^6 packaging cells of the ΨCRIP-P131 cell line (see Dranoff, G. et al., Proc. Natl. Acad. Sci. USA, 90, 3539-3543, 1993; and for the ΨCRIP-P131 cell line, see Wakimoto, H. et al., Jp. J. Cancer Res. 88, 296-305, 1997) were inoculated in a 10 cm dish.

After 24 hours, the culture medium of the BOSC 23 cells was filtered through a 0.45 or 0.22 µm syringe

filter, and Polybrene (hexadimethrine bromide, SIGMA H-9263) was added so as to give a final concentration of 8 μg/ml. 5 ml of this culture medium was added to the cells of the ΨCRIP-P131 cell line and incubated. After 16 hours' incubation, 5 ml of fresh Dulbecco's medium (complete) was added and the incubation was further continued overnight. The resulting ΨCRIP producer cells were cultured for 7 days in Dulbecco's medium (complete) containing 10 μg/ml of blasticidin (Funakoshi) to establish SCRIP/crmA.

For purposes of infection, 293 cells were inoculated in a dish on the day before infection. As soon as about 80% confluency was reached, 5 ml of the supernatant obtained by filtering the culture medium of the aforesaid ΨCRIP/crmA producer cells (which had been replaced by a fresh medium before 24 hours) and adding 8 μg/ml of Polybrene was added thereto.

After 16 hours' incubation, 5 ml of fresh Dulbecco's medium (complete) was added thereto and the incubation was further continued overnight. The resulting 293 cells were cultured for 7 days in Dulbecco's medium (complete) containing 10 μg/ml of blasticidin (Funakoshi), and then cultured for about 10 days in Dulbecco's medium (complete) containing no blasticidin to establish 293/crmA. Thus, there was obtained an apoptosis-resistant 293/crmA cell line. This 293/crmA cell line is resistant to transfection with plasmid DNA (e.g., pAxCAhFasL) capable of expressing an apoptosis-associated gene, as shown in Fig. 1. On the basis of this property, vectors can be generated as will be described later.

The base sequence of the plasmid pRx-ires-bsr used in this example is shown as SEQ ID NO:2. In this sequence, a base sequence consisting of a large number of "A"s arranged on the outside of the LTR of the retrovirus represents an unidentified sequence part for

convenience' sake. The plasmid pRx-ires used in this example is reported in Wakimoto, H. et al., Jp. J. Cancer Res. 88, 296-305, 1997. The bsr gene used in this example was generously supplied by Prof. Junichi Fujisawa, Department of Microbiology, Kansai Medical College.

Example 2: Generation of an apoptosis-resistant virus-sensitive cell line 293/bcl-xl

a) cDNA clone of Bcl-xl:

The base sequence of the cDNA of Bcl-xl is described in Boise, L.H. et al., Cell 74(4), 597-608 (1993).

In this example, pSKIIhBcl-xl generously supplied by Dr. Andy Minn, University of Chicago, Gwen Knapp Center for Lupus and Immunology Research (Fax 773-702-1576) was used for the cDNA of Bcl-xl. Of the reported cDNA base sequence, the nt135-nt836 translational region (about 700 bp) is contained in this plasmid. However, the base sequences of the parts upstream and downstream thereof are unknown because no information about it has been obtained from Dr. Andy Minn.

b) Construction of the plasmid pCA-Bcl-xl:

An *Eco*RI fragment (about 770 bp) containing hBcl-xl was excised from pSKIIhBcl-xl and subcloned into the *Eco*RI site of pCAcc to generate pCA-Bcl-xl. For pCAcc, see Yoshida Y et al., Biochem. biophys. Res. Commun. 230, 426-430 (1997).

c) Construction of pRx-Bcl-xl-bsr:

This *Eco*RI fragment was integrated into the *Eco*RI site of pRx-bsr. Thus, pRx-Bcl-xl-bsr containing the fragment in the right direction was obtained. The base sequence thereof is shown as SEQ ID NO:3.

In this sequence, a base sequence consisting of a large number of "A"s arranged on the outside of the LTR of the retrovirus represents an unidentified sequence part for convenience' sake. Moreover, the base

sequences of the parts upstream and downstream of the cDNA of Bcl-xl are conveniently represented by AAAAAAAAAA (i.e., ten "A"s) because their ranges are unknown.

d) Generation of ΨCRIP-P131/Bcl-xl:

A basic method for gene transfer by use of a retrovirus vector is described in Shinoura and Hamada, "Retroviruses", pp. 58-62; and Saito and Sugano (eds.), "An Extra Issue of Experimental Medicine: Experimental Methods for Gene Transfer and Expression Analysis", 1997, Yodosha. For the ΨCRIP-P131 cell line, see the aforementioned article by Wakimoto H. et al. Also in this example, the method described in the above references was basically employed to establish a cell line capable of expressing Bcl-xl on a high level. However, the method employed in this example was based on selection with blasticidin.

The transfection of BOSC 23 cells with the plasmid DNA of pRx-Bcl-xl-bsr was performed in the same manner as previously described for pRx-crmA-bsr. The method for infecting ΨCRIP-P131 cells with the retrovirus produced by the BOSC 23 cells was also the same as previously described for pRx-crmA-bsr. In this case, however, ΨCRIP-P131 cells infected with the retrovirus derived from pRx-Bcl-xl-bsr were selected with blasticidin. For the purpose of selection, ΨCRIP producer cells infected with the retrovirus derived from the BOSC 23 cells were cultured for 7 days in Dulbecco's medium (complete) containing 10 μg/ml of blasticidin (Funakoshi). Thus, a ΨCRIP-P131 cell line infected with the retrovirus derived from pRx-Bcl-xl-bsr was generated and named ΨCRIP-P131/Bcl-xl.

e) Establishment of the 293/Bcl-xl cell line:

The method for infecting 293 cells of the parent cell line with the retrovirus produced by ΨCRIP-P131/Bcl-xl cells was the same as previously described for the

establishment of the 293/crmA cell line. In this case, however, 293 cells infected with the retrovirus were selected with blasticidin. For the purpose of selection, 293 producer cells infected with the retrovirus derived from ΨCRIP-P131/Bcl-xl cells were cultured for 7 days in Dulbecco's medium (complete) containing 10 μg/ml of blasticidin (Funakoshi). Thus, a 293 cell line infected with the retrovirus derived from ΨCRIP-P131/Bcl-xl was established and named the 293/Bcl-xl cell line.

Example 3: Generation of an apoptosis-resistant virus-sensitive cell line 293/Bcl-2

a) The cDNA clone of Bcl-2 used in this example was obtained from the plasmid pB4 bcl-2a. The plasmid pB4 bcl-2a was generously supplied by Dr. Tsujimoto, Department of Medicine, Osaka University. The base sequence of an *Eco*RI fragment thereof is shown as SEQ ID NO:4.

b) Construction of pRx-Bcl 2-i-hCD 25:

This *Eco*RI fragment was made blunt-ended with T4 DNA polymerase (purchased from NEB Co.) and integrated into the blunt-ended *Cla*I and *Not*I sites of the plasmid pRx-ires-hCD 25 to generate pRx-Bcl 2-i-hCD 25. The base sequence thereof is shown as SEQ ID NO:5.

In this sequence, a base sequence consisting of a large number of "A"s arranged on the outside of the LTR of the retrovirus represents an unidentified sequence part for convenience' sake. The plasmid pRx-ires-hCD 25 used in this example is described in Shinoura and Hamada, "Retroviruses", pp. 58-62; and Saito and Sugano (eds.), "An Extra Issue of Experimental Medicine: Experimental Methods for Gene Transfer and Expression Analysis", 1997, Yodosha.

e) Generation of ΨCRIP-P131/Bcl-2:

A basic method for gene transfer by use of a retrovirus vector is described in Shinoura and Hamada,

"Retroviruses", pp. 58-62; and Saito and Sugano (eds.), "An Extra Issue of Experimental Medicine: Experimental Methods for Gene Transfer and Expression Analysis", 1997, Yodosha. For the ΨCRIP-P131 cell line, see the aforementioned article by Wakimoto H. et al. Also in this example, the method described in the above references was basically employed to establish a cell line capable of expressing Bcl-2 on a high level. However, the method employed in this example was based on selection by the expression of human CD 25.

The transfection of BOSC 23 cells with the plasmid DNA of pRx-Bcl 2-i-hCD 25 was performed in the same manner as previously described for pRx-crmA-bsr. The method for infecting ΨCRIP-P131 cells with the retrovirus produced by the BOSC 23 cells was also the same as previously described for pRx-crmA-bsr. In this case, however, ΨCRIP-P131 cells infected with the retrovirus derived from pRx-Bcl 2-i-hCD 25 were selected by staining the cells with human CD 25 antibody (FITC-labelled anti-Tac; purchased from Pharmingen Co.). Then, using a cell sorter (FACStar; Becton-Dickinson Co.), CD 25-positive cells were selected in the usual manner. Thus, a ΨCRIP-P131 cell line infected with the retrovirus derived from pRx-Bcl 2-i-hCD 25 was generated and named ΨCRIP-P131/Bcl 2.

d) Establishment of the 293/Bcl-xl cell line:

The method for infecting 293 cells of the parent cell line with the retrovirus produced by ΨCRIP-P131/Bcl 2 cells was the same as previously described for the establishment of the 293/crmA cell line. In this case, however, 293 cells infected with the retrovirus were selected by staining the cells with human CD 25 antibody (FITC-labelled anti-Tac; purchased from Pharmingen Co.). The method for selecting CD 25-positive cells was the same as described above for the selection of ΨCRIP-P131/Bcl 2. Thus, a 293 cell line infected with the retro-

virus derived from ΨCRIP-P131/Bcl 2 was established and named the 293/Bcl 2 cell line.

Example 4: Construction of a pAx cosmid pAxCAhFAS

a) Preparation of hFAS cDNA:

hFAS cDNA was prepared in the following manner. Cells of the human monocyte-derived cell line THP1 (ATCC) purchased from ATCC were treated with 50 ng/ml of a phorbol ester (PMA; purchased from Sigma Co.) for 24 hours, and mRNA was prepared from these cells in the usual manner. Using this mRNA as a template, RT-PCR (reverse transcription-polymerase chain reaction) was performed in the usual manner to obtain about 1028 bp cDNA. The primers used in this PCR (polymerase chain reaction) were the following oligonucleotides which had been prepared in our laboratory.

#457 CCGAATTCTCGAGCACCATGCTGGGCATCTGGACCCTC
(SEQ ID NO:6)

#458 CCGAATTCTAGACCAAGCTTTGGATTTCATTTCTGAAGTTTGA
(SEQ ID NO:7)

b) Confirmation of the base sequence of cDNA:

The *Eco*RI fragment of the resulting cDNA was cloned into the *Eco*RI site of pBluescriptKS+ (pKS) (purchased from Stratagene Co.). Thereafter, a clone pKShFAS having a *Kpn*I site located upstream of the 5' end of hFAS was used. The base sequence of the cDNA of pKShFAS was determined according to the Sanger method and confirmed to be identical to that reported in GenBank.

c) Construction of a plasmid pCEPhFAS:

A *Kpn*I/*Not*I fragment (about 1100 bp) was excised from pKShFAS and subcloned into the *Kpn*I/*Not*I sites of pCEP 4 (purchased from Invitrogen Co.) to generate pCEPhFAS.

d) Construction of the plasmid pCAhFAS:

An *Eco*RI fragment (about 1028 bp) containing hFAS was excised from pCEPhFAS and subcloned into the

*Eco*RI site of pCAcc to generate pCAhFAS. For pCAcc, see Niwa, H., Yamamura, K., and Miyazaki, J. (1991), Gene (Amst.) 108, 193-200; and Yoshida Y et al., Biochem. Biophys. Res. Commun. 230, 426-430 (1997).

e) pAxCAhFAS:

A *Cla*I fragment (3.3 kbp) was excised from pCAhFAS and subcloned into the *Cla*I site of pAdex1cw. A clone having the "leftward" direction was selected and regarded as pAxCAhFAS. For the pAx cosmid, see, for example, Sato et al., Methods for the Generation of Recombinant Adenoviruses, pp. 27-42; Saito and Sugano (eds.), "An Extra Issue of Experimental Medicine: Experimental Methods for Gene Transfer and Expression Analysis", 1997, Yodosha; or Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C., and Saito, I. (1996), Proc. Natl. Acad. Sci. USA 93, 1320-1324.

Example 5: Construction of a pAx cosmid pAxCAhFasLig

A cosmid pAxCAhFasLig was constructed in substantially the same manner as described above for pAxCAhFAS. The cDNA of hFasLig (human FAS ligand) used in this example was derived from the plasmid pFLNL4 generously supplied by Dr. E. Mita, First Department of Internal Medicine, Osaka University [Mita E, Biochem. Biophys. Res. Commun. 204(2), 468-474 (1994)]. The base sequence of the aforesaid cDNA is shown in SEQ ID NO:8.

A *Hind*III/*Nsi*I fragment was excised from this plasmid and subcloned into the *Hind*III/*Pst*I sites of pBluescriptSKII (hereinafter abbreviated as pSK; purchased from Stratagene Co.) to generate pSKII+FasLig. Then, a DNA fragment containing FasLig was excised from pSKII+FasLig by *Cla*I/*Xba*I cleavage, made blunt-ended with T4 DNA polymerase, and ligated to the *Eco*RI site of pCAcc which had been made blunt-ended with T4 DNA polymerase. Moreover, the direction of the fragment was confirmed to generate pCAhFasLig. Thereafter, a DNA

fragment containing FasLig was excised from pCAhFasLig by *Cla*I cleavage, and cloned into the *Cla*I site of pAxcw to generate pAxCAhFasLig.

Example 6: Construction of a pAx cosmid pAxCAhFLICE (Caspase-8)

A cosmid pAxCAhFLICE (Caspase-8) was constructed in substantially the same manner as described above for pAxCAhFAS. The cDNA of hFLICE (Caspase-8) used in this example was that described in Muzio et al., Cell 85, 817-827 (1996).

Specifically, pcDNA 3 containing the cDNA of hFLICE (Caspase-8) was obtained from Dixit VM et al. A DNA fragment containing FLICE was excised from this plasmid by *Hind*III/*Not*I cleavage, made blunt-ended with T4 DNA polymerase, and ligated to the *Eco*RI site of pCAcc which had been made blunt-ended with T4 DNA polymerase. Moreover, the direction of the fragment was confirmed to generate pCAhFLICE. Then, a DNA fragment containing FLICE was excised from pCAhFLICE by *Cla*I cleavage, and cloned into the *Cla*I site of pAxcw to generate pAxCAhFLICE.

Example 7: Construction of a pAx cosmid pAxCAhBcl-xs

A cosmid pAxCAhBcl-xs was constructed in substantially the same manner as described above for pAxCAhFAS. The cDNA of pAxCAhBcl-xs used in this example was pSKIIhBcl-xl generously supplied by Dr. Andy Minn, University of Chicago, Gwen Knapp Center for Lupus and Immunology Research (Fax 773-702-1576) for the cDNA of Bcl-xl.

A plasmid pCA-Bcl-xs was generated by excising an *Eco*RI fragment (about 600 bp) containing hBcl-xs from pSKIIhBcl-xs and subcloning this fragment into the *Eco*RI site of pCAcc. Then, a DNA fragment containing CA-Bcl-xs was excised from pCA-Bcl-xs and cloned into the *Cla*I site of pAxcw to generate pAxCA-Bcl-xs.

Example 8: Construction of a pAx cosmid pAxCAhFADD

A cosmid pAxCAhFADD was constructed in substantially the same manner as described above for pAxCAhFAS. The cDNA of pAxCAhFADD used in this example was that described in Muzio et al., Cell 85, 817-827 (1996).

Specifically, pcDNA 3 containing the cDNA of hFADD was obtained from Dixit VM et al. A DNA fragment (about 700 bp) containing hFADD was excised from this plasmid by *Hind*III/*Xho*I cleavage, made blunt-ended with T4 DNA polymerase, and ligated to the *Eco*RI site of pCAcc which had been made blunt-ended with T4 DNA polymerase. Moreover, the direction of the fragment was confirmed to generate pCAhFADD. Then, a DNA fragment (about 3 kbp) containing CAhFADD was excised from pCAhFADD by *Cla*I cleavage, and cloned into the *Cla*I site of pAxcw to generate pAxCAhFADD.

Example 9: Generation of an Ax recombinant adenovirus AxCAhFAS

a) The DNA of the cosmid pAxCAhFAS was prepared in the usual manner and used to transfect 293/crmA cells. Then, clones of the desired adenovirus AxCAhFAS were selected in the usual manner. For the method for generating the adenovirus, see, for example, Sato et al., Methods for the Generation of Recombinant Adenoviruses, pp. 27-42; Saito and Sugano (eds.), "An Extra Issue of Experimental Medicine: Experimental Methods for Gene Transfer and Expression Analysis", 1997, Yodosha; or Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C., and Saito, I. (1996), Proc. Natl. Acad. Sci. USA 93, 1320-1324.

b) The function of the resulting adenovirus AxCAhFAS was examined in the following manner.

(Procedure) Using 293/crmA cells, a tertiary seed of the recombinant adenovirus AxCAhFAS was prepared and used to infect human glioma cells of the U251 cell line (generously supplied by NCI, U.S.A.) at an moi of

100. After 2 days, the expression of FAS was confirmed by FACS. FACS was performed according to the standard method described, for example, in K. Hanada et al., J. Leukocyte Biology 60, 181-190, 1996. As a control, U251 cells were infected with AxCAlacZ at an moi of 100. Anti-human CD 95-FITC (Pharmingen) was used as the primary antibody in an amount of 10 µl per 1 x 10^6 cells, and anti-Leu2a (anti-CD 8)-FITC (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used as a control antibody in an amount of 10 µl per 1 x 10^6 cells.

(Results) The results are shown in Fig. 2. It was confirmed that the expression of FAS in U251 cells was very greatly enhanced by infection with the recombinant adenovirus AxCAhFAS (peak 4). Peak 1 shows U251 cells stained with the control antibody. Peak 2 shows uninfected U251 cells stained with anti-human CD 95-FITC (Pharmingen). In U251 cells, a slight expression of FAS is observed. Peak 3 shows U251 cells infected with AxCAlacZ as a control. Infection with the simple adenovirus causes a slight change in the expression level of FAS, but this change is very slight as compared with the change shown by peak 4. The AxCAlacZ adenovirus used in this example is one commonly used as a control, and is described, for example, in Nakamura et al., Cancer Res. 54, 5747-5760, 1994. These results demonstrate that an intense expression of FAS is achieved by infection with the recombinant adenovirus AxCAhFAS.

Example 10: Generation of an Ax recombinant adenovirus AxCAhFasLig

a) The DNA of the cosmid pAxCAhFasLig was prepared in the usual manner and used to transfect 293/crmA cells. Then, clones of the desired adenovirus AxCAhFasLig were selected in the usual manner. The method for generating the adenovirus was the same as previously described for the generation of AxCAhFAS.

b) The function of the resulting adenovirus AxCAhFasLig was examined in the following manner.

(Procedure) Using 293/crmA cells, a tertiary seed of the recombinant adenovirus AxCAhFasLig was prepared and used to infect human glioma cells of the U373 cell line (purchased from ATCC) at an moi of about 100. After 2 days, the expression of FAS ligand was confirmed by FACS. FACS was performed according to the standard method described, for example, in K. Hanada et al., J. Leukocyte Biology 60, 181-190, 1996. Anti-Fas ligand (human)-biotin (Sumitomo Electric, Yokohama, Japan) was used as the primary antibody in an amount of 5 μl per 1 x 10^6 cells, and streptoavidin-PE (Pharmingen, San Diego, CA) was used as the secondary antibody in an amount of 0.5 μl per 1 x 10^6 cells.

(Results) The results are shown in Fig. 3. It was confirmed that the expression of FAS ligand in U373 cells was induced by infection with the FAS ligand adenovirus (AxCAhFasLig). Peak 1 shows the results of analysis of control U373 cells, and peak 2 shows the results of analysis of U373 cells infected with the FAS ligand adenovirus (AxCAhFasLig). These results demonstrate that an intense expression of FAS ligand is achieved by infection with the recombinant adenovirus AxCAhFasLig.

Example 11: Generation of an Ax recombinant adenovirus AxCAhBcl-xs

a) The DNA of the cosmid pAxCAhBcl-xs was prepared in the usual manner and used to transfect 293/crmA cells. Then, clones of the desired adenovirus AxCAhBcl-xs were selected in the usual manner. The method for generating the adenovirus was the same as previously described for the generation of AxCAhFAS.

Example 12: Generation of an Ax recombinant adenovirus AxCAhFADD

a) The DNA of the cosmid pAxCAhFADD was pre-

pared in the usual manner and used to transfect 293 cells. Then, clones of the desired adenovirus AxCAhFADD were selected in the usual manner. The method for generating the adenovirus was the same as previously described for the generation of AxCAhFAS. In the case of AxCAhFADD, viruses up to the secondary seed could be prepared by using 293 cells of the parent cell line instead of 293/crmA cells. However, for viruses of the tertiary seed and further, 293 cells of the parent cell line failed to achieve a sufficiently high viral titer. Accordingly, for viruses of the tertiary seed and further, a virus suspension was prepared by using 293/crmA cells. (Thus, with respect to an adenovirus capable of expressing an apoptosis-associated gene, it may happen that, even if the recombinant virus can be generated by using ordinary 293 cells, the preparation of a large amount of the virus induces apoptosis in 293 cells of the parent cell line and fails to achieve a sufficiently high viral titer. In such a case, it is expected that use of the 293/crmA cell line or the like permits the preparation of a large amount of the virus. This is also a useful application of the 293/crmA cell line and like cell lines disclosed herein.) For the definition of secondary and tertiary seed viruses, see, for example, Sato et al., Methods for the Generation of Recombinant Adenoviruses, pp. 27-42; or Saito and Sugano (eds.), "An Extra Issue of Experimental Medicine: Experimental Methods for Gene Transfer and Expression Analysis", 1997, Yodosha.

b) The functions of the resulting adnovirus AxCAhFADD were confirmed by the fact that, when a subline of the human glioma cell U251 (i.e., U251-FAS-H which had been established in out laboratory) was infected therewith at MOI = 5, a marked cell death (apoptosis) was induced. Fig. 4 shows the state of the subline of U251 (U251-FAS-H) two days after infection

with AxCAhFADD. Although U251 cells inherently grow while adhering to a plastic plate, most of the cells infected with AxCAhFADD were found floating on the surface. The functions of the adnovirus AxCAhFADD were also confirmed by infecting the human glioma cell U373 therewith at MOI = 100 and subjecting the proteins extracted therefrom on the second day to western blot analysis. Fig. 5 shows the results of western blot analysis of the proteins extracted from uninfected control U373 cells (lane 1), control U373 cells infected with AxCA-LacZ at MOI = 100 (lane 2), and U373 cells infected with AxCAhFADD at MOI = 100 (lane 3). These analyses were carried out according to an ordinary procedure using a commercially available anti-FADD antibody.

Other References:

Unless otherwise indicated, the general methods employed in the practice of the present invention are the standards methods described in the following protocols.

1. Coligan JE et al. (eds.), Current Protocols in Immunology, published by John Wiley and Sons, Inc.

2. Ausubel FM et al. (eds.), Current Protocols in Molecular Biology, published by John Wiley and Sons, Inc.

3. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. ed., 1989, Cold Spring Laboratory Press, New York.

Effects of the Invention

According to the present invention, the generation of adenovirus vectors capable of expressing apoptosis-associated genes, which has been impossible in the prior art, can be achieved by providing, for example, an apoptosis-resistant 293 producer cell line and using it.

The recombinant adenoviruses capable of apoptosis-associated genes, which are generated in the above-described manner, can be applied to the treatment of various diseases.

Moreover, the method disclosed herein is believed to be useful in the generation of AxCA-(objective gene) adenoviruses capable of expressing various other apoptosis-associated genes and cell cycle-associated genes on a high level.

It is a matter of course that the usefulness of this method is not limited to 293/crmA.

Even if various other apoptosis resistance genes such as bcl-2, bcl-xs, FLIP (homo sapience FLICE-like inhibitory protein, short form and long form; Irmler et al., Nature 388, 190-195, 1997) and ILP (IAP-like protein ILP; Duckett et al., EMBO J. 15(11), 2685-2694, 1996) are introduced (stably or transiently) into 293 cells, the resulting cells are believed to be useful in the generation of AxCA-(objective gene) adenoviruses capable of expressing various other apoptosis-associated genes (such as FAS, FAS-Lig and FLICE) and cell cycle-associated genes on a high level.

Similarly, it is expected that the generation of viruses other than adnoviruses, and other microorganisms or their products (e.g., proteins), which has been difficult because of their high cytotoxicity to host cells, may become possible. This method can be applied to various fields including the generation of vectors for gene therapy and the preparation of vaccines against viruses and other microorganisms or their products (e.g., proteins).

Furthermore, the AxCA-(objective gene) adenoviruses capable of expressing various apoptosis-associated genes and cell cycle-associated genes on a high level, which are generated by the method disclosed herein, are believed to be useful in the following cases:

(1) Induction of apoptosis by gene transfer.

(1-1) Application to the treatment of cancer by destroying cancer cells selectively. Apoptosis induction therapy for cancer cells.

(1-2) Utilization in the treatment of autoimmune diseases and graft rejection reactions. Apoptosis induction therapy for immunocompetent cells.

(1-3) Apoptosis induction therapy for inflammatory cells in inflammatory diseases.

(2) Utilization of the inhibition of apoptosis by gene transfer. In this case, it may be possible to inhibit harmful apoptotic reactions, for example, by expressing bcl-2, bax (antisense or dominant-negative), FAS (antisense or dominant-negative), FADD (antisense or dominant-negative) or the like on a high level.

(2-1) In fulminant hepatitis, the inhibition of apoptosis by gene transfer to hepatic cells may be utilized to minimize a severe necrosis of hepatic cells and thereby save the patient's life.

(2-2) The prevention and treatment of autoimmune diseases, graft rejection reactions and other diseases in which harmful apoptosis is considered to be occur.

(2-3) The prevention and treatment of inflammatory diseases and other diseases in which harmful apoptosis is considered to be occur.

(2-4) Nervous diseases. Apoptosis may be controlled to promote the regeneration and differentiation of nerves. Moreover, harmful apoptosis of nerve cells, which is observed in traumata, angiopathy and degenerative diseases (e.g., Alzheimer's disease), may be controlled to arrest or treat these nervous diseases.

CLAIMS

1. An apoptosis-resistant virus-sensitive cell line having an apoptosis resistance gene introduced thereinto.

2. A cell line as claimed in claim 1 wherein the apoptosis resistance gene is selected from the group consisting of the crmA, bcl-2, bcl-xl, FLIP, survivin, IAP and ILP genes.

3. A cell line as claimed in claim 1 or 2 wherein the virus-sensitive cell line is the 293 cell line derived from a human fetal kidney.

4. A cell line as claimed in any of claims 1 to 3 wherein the virus is selected from the group consisting of viruses belonging to the families *Adenoviridae*, *Retroviridae*, *Parvoviridae*, *Herpesviridae*, *Poxviridae*, *Papovaviridae* and *Hepadnaviridae*.

5. A cell line as claimed in claim 3 wherein the virus is a human adenovirus belonging to the family *Adenoviridae*.

6. A method for the generation of a recombinant virus containing an apoptosis-associated gene which comprises the steps of:

(A) providing an apoptosis-resistant virus-sensitive cell line having an apoptosis resistance gene introduced thereinto;

(B) transfecting cells of the cell line of step (A) with a virus (or expression vector) containing an apoptosis-associated gene in combination with a helper virus, if necessary; and

(C) culturing the transfected cells of step (B) to produce a recombinant virus containing the apoptosis-associated gene.

7. A method as claimed in claim 5 wherein the apoptosis-associated gene is selected from the group consisting of the FAS, Fas Lig, FLICE and bcl-xs genes.

8. A method as claimed in claim 6 or 7 wherein the virus-sensitive cell line is the 293 cell line derived from a human fetal kidney.

9. A method as claimed in any of claims 6 to 8 wherein the virus is selected from the group consisting of viruses belonging to the families *Adenoviridae*, *Retroviridae*, *Parvoviridae*, *Herpesviridae*, *Poxviridae*, *Papovaviridae* and *Hepadnaviridae*.

10. A recombinant virus containing an apoptosis-associated gene which is generated by a method as claimed in any of claims 6 to 9.

11. A recombinant adenovirus containing an apoptosis-associated gene.

12. A recombinant adenovirus as claimed in claim 11 wherein the apoptosis-associated gene is selected from the group consisting of the FAS (CD 95), Fas Lig, FLICE, Bax, bcl-2, bcl-2 antisense and bcl-xs genes.

Fig. 1

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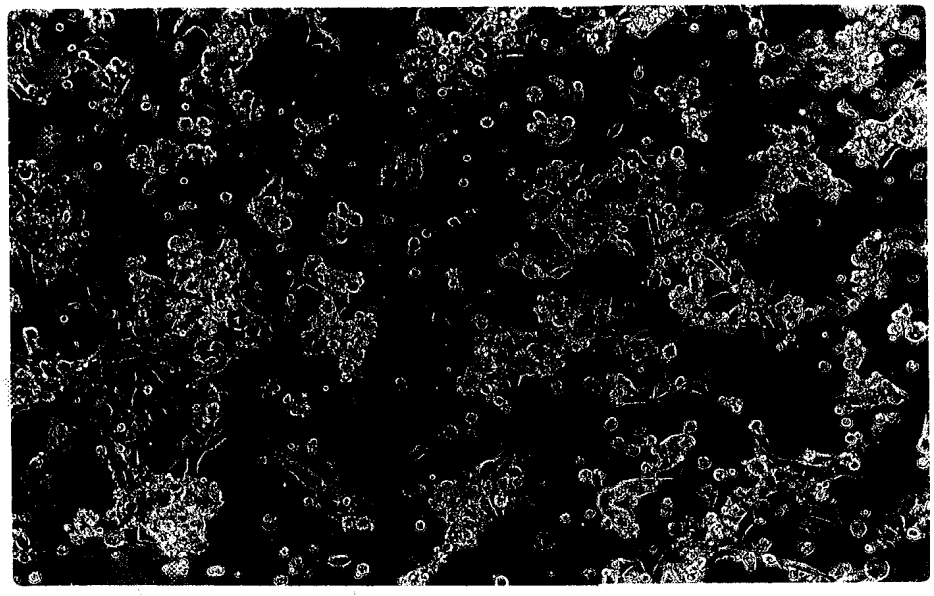

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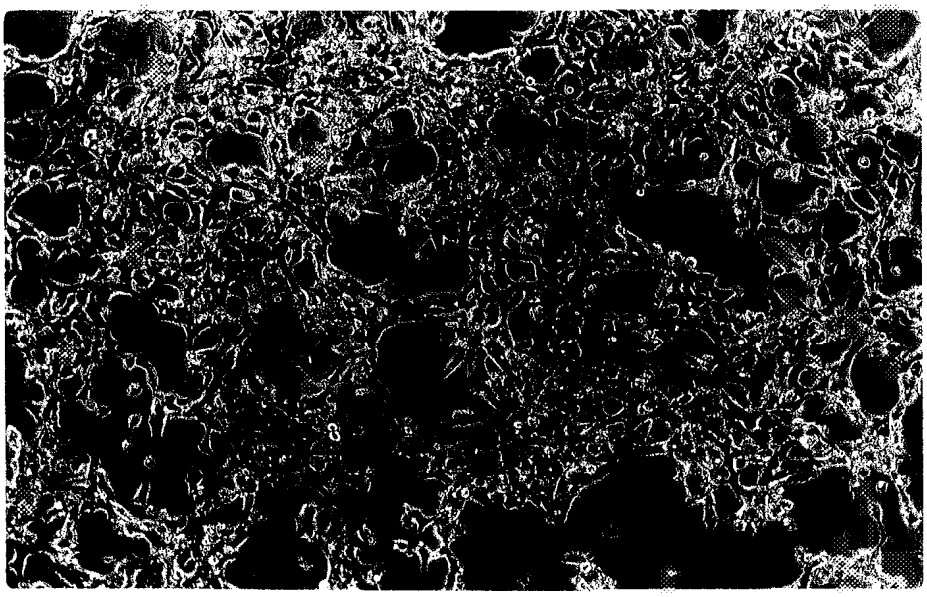

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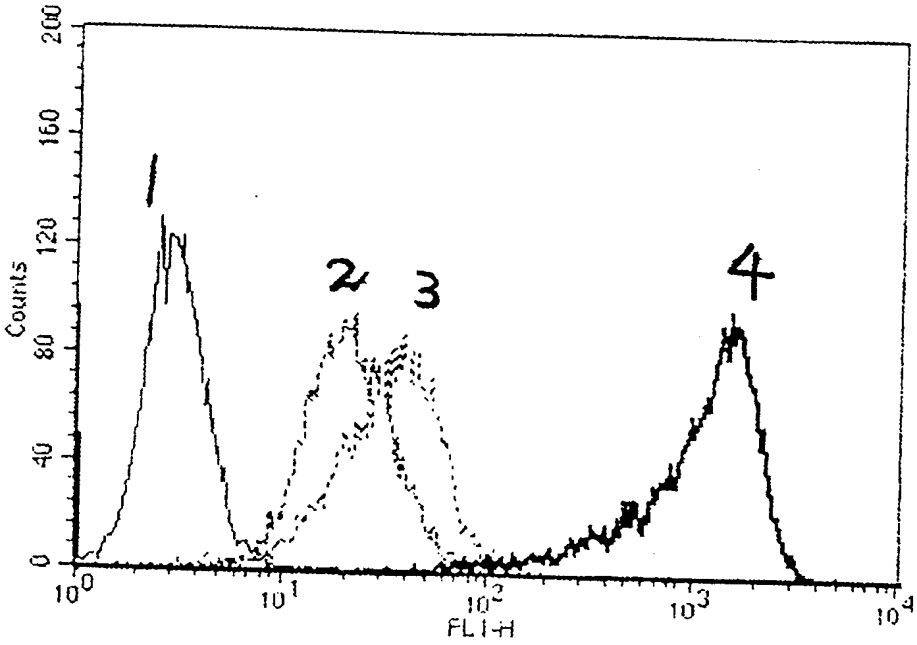


Fig. 3

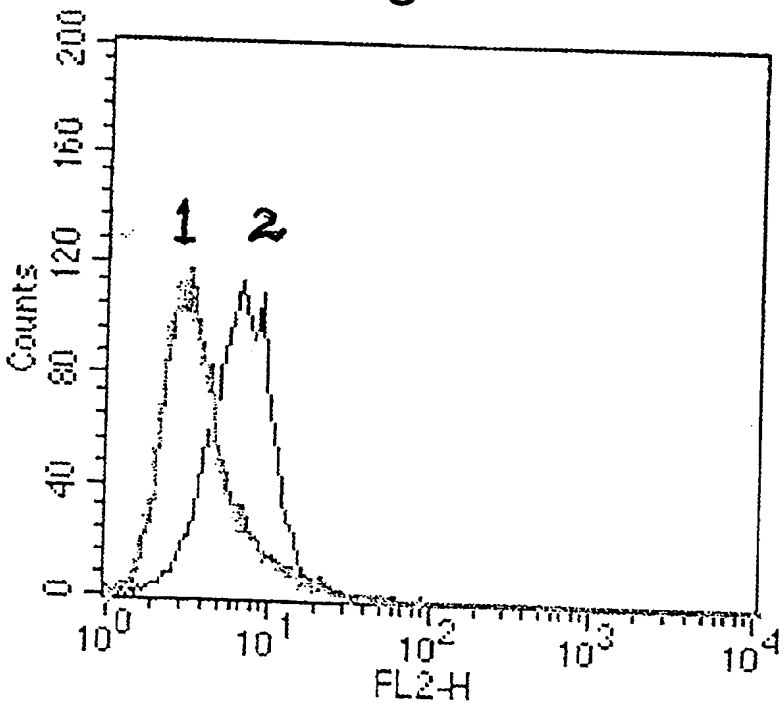


Fig. 4

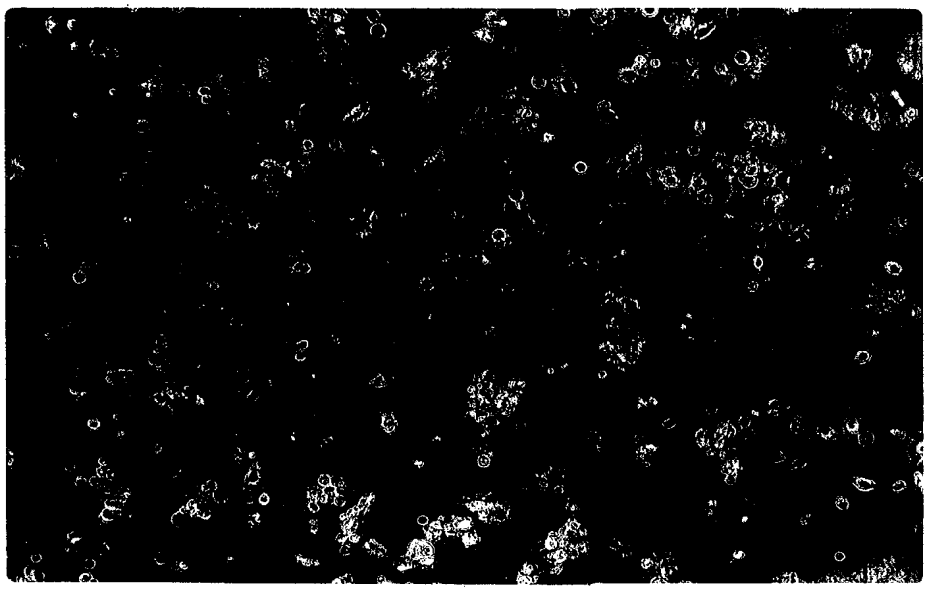
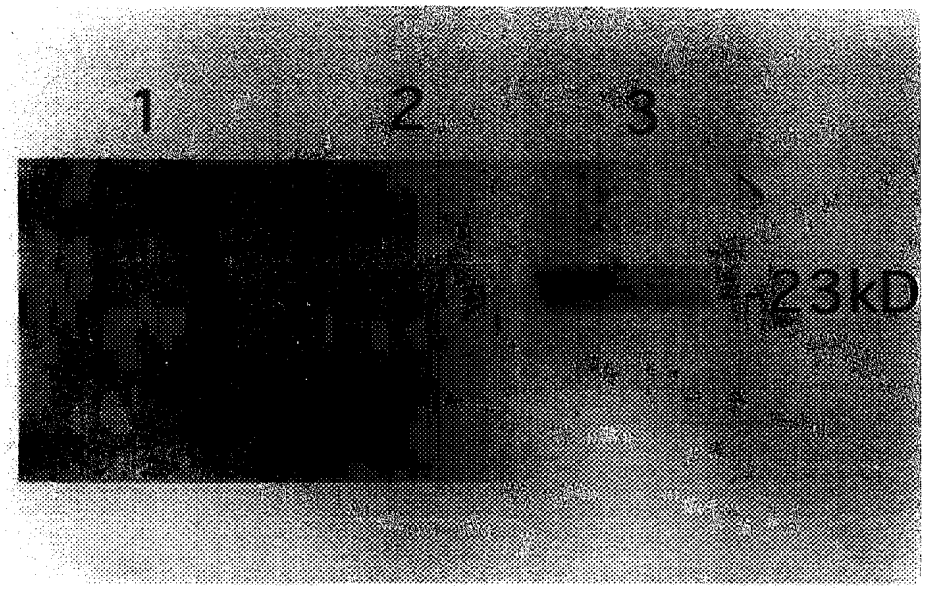


Fig. 5



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